Making it big : how characean algae use cytoplasmic streaming to enhance transport in giant cells

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Colloquially known as *stoneworts* after the lime depositions on their surface, the characean algae (fig 2.1) are a family of weeds belonging to the order *Charales*. Found in fresh and brackish waters, these multicellular algae have the appearance of plants, growing in thin segmented shoots that sprout whorls of branchlets every few centimetres (fig 2.1a). A remarkable feature of these organisms is that the *internodal* 'segments' of the shoots are in fact giant *single* cells. With diameters up to 1 mm and lengths that can exceed 10 cm, these cells are among the largest known in existence.

Inside the internodal cells there is an active transportation system. The surface of the cell is divided into two band-shaped domains, along which the fluid is transported up and down at rates of up to 100 μm/s (fig 2.1c). Because the upward and downward flowing bands tend to spiral around each other, this type of circulation is sometimes called *rotational streaming*. As we will discuss in this chapter, cytoplasmic streaming is known to play a role in a number of metabolic processes. It enhances transport between cells (Ding and Tazawa, 1989), has been shown to be necessary for the formation of alkaline bands which aid carbon uptake, and is widely been hypothesised to play a role in intracellular transport and mixing (Hochachka, 1999; Pickard, 2003, 2006). However despite the fact that streaming has been implicated in a range of metabolic processes, the precise ways in which it aids the organism remain poorly understood.

The central focus of our research in this area is to quantify fluid dynamical aspects of this circulation and elucidate its role in enhancing metabolic rates. As discussed in the previous chapter, the very size of the these cells suggests that diffusion should be expected to be prohibitively slow. Moreover, the myosins found in these species are amongst the fastest in existence. This suggests that there must be some form of proportional benefit to the high rates of circulation observed in characean internodes.

In this chapter we review the research performed on the Charales over the last decades. While the focus of this review lies on work pertinent to the function of cytoplasmic streaming, our scope will be fairly broad to allow the reader to get a sense of the range of processes in which streaming may be implicated, as well as the interconnectedness of many of these features. The first part of this review will discuss some aspects of growth mechanics and electrophysiology as studied in these species. In the second part we will present an overview of the studies on streaming.

2.1 Morphology and Life Cycle

Characean algae grow in thin shoots (fig 2.1a) anchored in the substrate with root-like *rhizoidal* cells. Reflecting the terminology commonly used for vascular plants, the branching points of the stem are known as *nodes*, whereas the segments of the stem connecting these points are called *internodes*. While the internodes are giant single cells, the nodes are complex multicellular structures. A whorl of ‘branchlets’ forms at the nodes, 6-16 in number depending on the species. The constituent cells of these branchlets are sometimes called ‘leaf’ cells. They are highly similar to the internodal cells, though they are typically shorter in length and often do not exhibit the spiralling structure characteristic of the internodes. It is also at the node that reproductive structures (fig 2.1e) form.

The most studied part of this organism is without doubt the characean internode (figure 2.1b), a single cylindrical cell with a diameter up to 1 mm and a length that can exceed 10 cm. Like most plant cells, the internode is enclosed by a cellulose cell wall. A layer of *cytoplasm* of roughly 10 μm in thickness lines the inside of the cell wall. The cytoplasm is separated by a membrane known as the *tonoplast* from the central vacuole that occupies the bulk of the cellular volume. This vacuole fulfils a multitude of metabolic roles, acting as storage compartment for sugars, polysaccharides, and organic acids, sequestering toxins such as heavy metals, and functioning as a buffering reservoir that helps to maintain ionic and pH homoeostasis in the cytoplasm (Taiz, 1992). Additionally, the vacuole holds a 0.13 M concentration of salts (Tazawa, 1964), producing an outward osmotic pressure equivalent to 5 bars that lends the cell its rigidity.

The characean cytoplasm exhibits many structures that are common to higher plants, including the Golgi apparatus, Endoplasmic Reticulum (ER) and cytoskeletal structures. *Chloroplasts*, the organelles responsible for photosynthesis, are packed into helical rows that spiral along the cellular surface (fig 2.1d). The chloroplasts are surrounded by a stagnant layer of fluid, sometimes called the *ectoplasm*, that houses most of the *mitochondria* in the cell.

On the inside of the chloroplast rows, bundled actin filaments act as tracks for myosins that drag structures within the cell (Kachar, 1985; Kachar and Reese, 1988) and thereby entraining the inner part of the cytoplasm, sometimes known as the *endoplasm*. With streaming rates as high as 100 μm/s, the myosin XI found in *Chara* is the fastest known in existence (Shimmen and Yokota, 2004). As a result of a reversed polarity of the

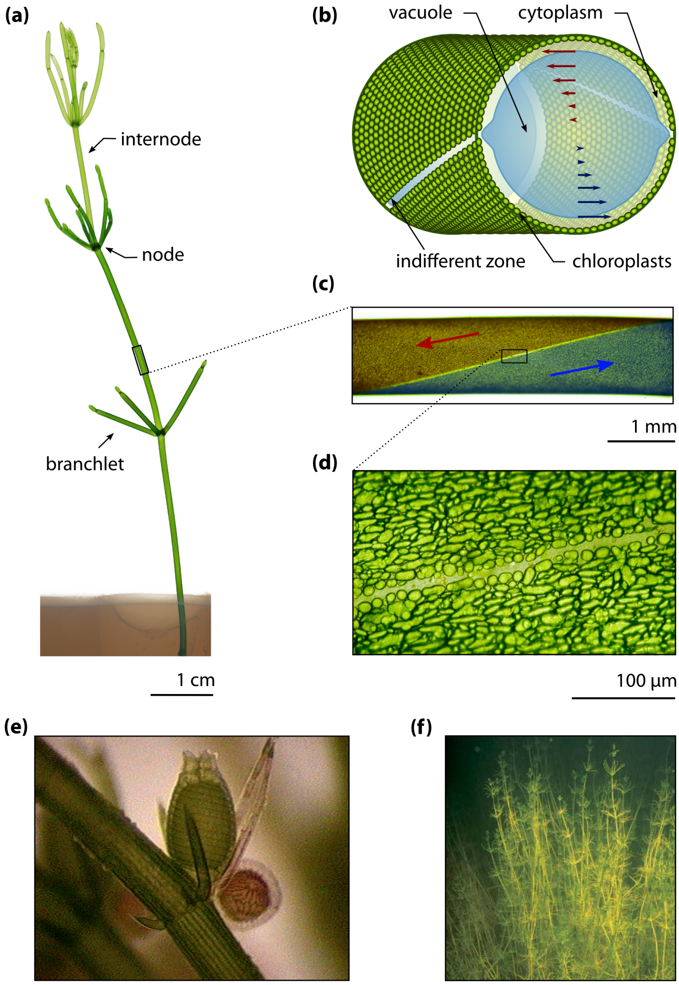

Figure 2.1 Anatomy of the characean algae. **(a)** A Chara Corallina shoot, showing *internodal* segments, connected by nodal structures sprouting a whorl of branchlets. The internodes are giant single cells several centimetres in length **(b)** Schematic cross-section of an internodal cell. An outer layer of cytoplasm moves up and down along the cell in two bands separated by a *neutral line*. The movement in the cytoplasm drives a shear flow in the central vacuole. **(c)** Enlarged microscopy image of an internodal cell, showing the spiralling neutral line that separates the two bands of streaming **(d)** Further enlarged image showing the packing of chloroplasts around the neutral line. **(e)** Reproductive structures in Chara Zeylanica (*image: http://bib18.ulb.ac.be/*). The ovaloid *oogonium* is the female structure, whereas sperm production originates in the round *antheridium*. **(f)** Shoots of Chara Hispida in an underwater meadow (*image: Sue Scott*).

actin filaments, the cytoplasm is organised in two bands flowing in opposite directions. These bands tend to spiral around each other, producing a "barber-pole" velocity at the cell periphery (fig 2.1c-d). The two interface lines between these bands is are known as *neutral lines*, or *indifferent zones*. The neutral lines are marked by the absence of chloroplasts (fig 2.1d) and visible as two light lines crossing the cell surface.

The internodes connect to the nodal structures through by means of channels known as *plasmodesmata*. These channels are bridges of cytoplasm between cells. The plasmodesmata in Charales are similar to those found in higher plants, but may have evolved separately (Franceschi et al., 1994). Most notably, in higher plants the endoplasmic reticulum is known to extend through plasmodesmata, but characean algae lack this feature. The generally accepted size limit for these channels is 800-1000 Da (Lucas et al., 1993) for diffusive transport, though molecules as large as 45 kDa have been shown to move between cells on longer time scales (Kikuyama et al., 1992).

2.1.1 Habitat

The Charales typically make their habitat on the bottom of shallow lakes, ditches, and ponds. These species are known to achieve high rates of growth compared to vascular plants, particularly in low nutrient environments. As such, they are often among the first species to colonise a newly cleared area. Their growth can form dense meadows that dominate their habitat. The high biomass content in these meadows acts as a nutrient sink that helps keep waters clear, stabilising the environment to low trophic levels and reducing turbidity by preventing large phytoplankton growth (Kufel and Kufel, 2002; Blindow et al., 2002; Schwarz and Hawes, 1997). In more nutrient rich environments, characean algae tend to be overtaken by flowering plants. They have a low tolerance for pollution by phosphates and nitrates associated with widespread fertiliser usage. As a result of this many Charales species are currently considered endangered (Stewart, 2004).

The lime depositions from which the species get their name are a consequence of the alkalinity of their habitat. In an alkaline environment, dissolved carbon is predominantly available in the form of bicarbonate (HCO_3^-). In order to turn bicarbonate into CO_2 a proton is needed. For this reason, most Charales precipitate calcium carbonate. This precipitation yields the required proton which is then combined with bicarbonate

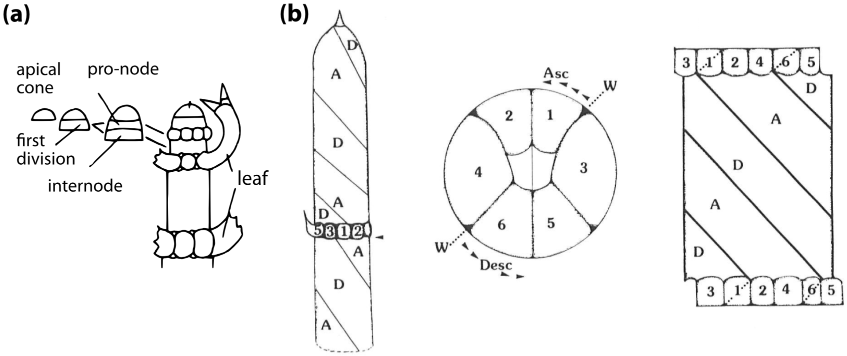

Figure 2.2 Cell organisation during tip growth. **(a)** Tip growth in Nitella Axillaris, as modified from Green (1958). A single cell at the top of the shoot, known as the *apical cone* divides once every few days. The newly formed cell then divides once more to form what will become the new node and internode. **(b)** Nodal arrangement of cells as reproduced from Shepherd and Goodwin (1992b). The pro-node first divides along the vertical line producing cells 1 and 2. Sub-sequent division of these cells produces the cells labelled 3 and 4, which then divide to form cells 5 and 6. The neutral lines of the internode below the nodal complex connect at the points marked (W). Once a new internode is formed by a subsequent division of the apical cone, its neutral lines connect at a position shifted with respect to the lower zone.

to produce water and CO_2 that can be used for photosynthesis. Over time, this accumulation produces rings of lime crust on the surface of the plant which in some cases cover the cell almost entirely.

2.1.2 Growth and Reproduction

In order to extend towards sunlight, plant cells expand their volume 10- to 20-fold over the course of development (Green et al., 1971; Taiz, 1992). The majority of this volume is occupied by the central vacuole, where high concentrations of salts are sequestered, resulting in large outward osmotic pressures that maintain the rigidity, or *turgor*, of the cell. Perhaps one of the most important functions of these vacuolar balloons is to serve as energetically favourable means of expanding the volume of the organism. While there is a considerable cost associated with the transport against electrochemical gradients required to maintain the solute concentration in the vacuole, it is much cheaper to expand cell size by water uptake than by protein synthesis (Raven, 1987; Taiz, 1992). With their exceptionally long internodal cells, whose vacuoles occupy about 95% of the cellular volume, characean algae represent an extreme case of this growth strategy. Since these species grow as weeds in low-nutrient conditions and can out-compete higher plants for lake dominance (Coops, 2002), the large cell size in these organisms, may be partially understood as result of competition for light in low nutrient conditions.

Characean algae can reproduce either by fragmentation, whereby a new plant develops out of a segment separated from a neighbouring shoot, or by the formation of spores. Reproduction by sporulation is sexual. The female reproductive structure is known as the *oogonium*, whereas the male organ is the called the *antheridium*. Some species have male and female plants, but in others such as Chara Zeylanica (fig 2.1e), both sexual structures are found on the same plant. A fertilised oogonium forms an *oospore*. In periods with unsuitable growing conditions these spores can remain viable for decades (Stewart, 2004).

Growth in characean algae occurs by repeated division of a single cell at the tip of the plant, known as the *apical cone* (fig 2.2). During shoot development this cell divides every few days. The interval of this division can be quite regular and is sometimes called the *plastochron*. After division, the newly formed cell divides once more into an upper *pro-node* and an internode (fig 2.2a). The internode undergoes a remarkable degree of expansion, increasing in volume by 5 orders of magnitude over a period of 2-3 weeks (Green, 1958).

The pro-node undergoes a number of subsequent divisions to form the nodal structure. In Chara Corallina, there is an initial division into 6 cells (fig 2.2) which connect to the ascending and descending streams in an specific manner (Shepherd and Goodwin, 1992b). In later stages these cells undergo further divisions to form a complex multicellular structure, though the initial symmetry is still reflected in the fact that Chara Corallina typically forms a whorl of 6 branchlets. It is also known that the reproductive structures are exclusively formed on the part of the node connecting to the descending stream.

2.2 A Model System in Plant Biology

Established as a genus by Linnaeus in 1753, Chara cells have been studied since the early days of microscopy (Corti, 1774). The species are now recognised as the closest living relatives of land plants (Kranz et al., 1995; Karol et al., 2001) and occupy a unique position in the evolutionary tree. Subcellular processes are highly developed and perform most functions common to higher plants. This high degree of similarity, along with the robustness of internodal cells under manipulation has allowed these organisms to function as a model system in a wide range of plant physiology research. The species are well known for studies performed on membrane transport and electrophysiology (Shimmen et al., 1994), turgor-driven cell wall expansion (Green et al., 1971; Proseus et al., 2000), and cytoskeletal organisation. Additionally, they have been used in studies on calcification and carbon fixation (Lucas, 1975), intercellular transport through plasmodesmata (Ding and Tazawa, 1989; Lucas, 1995) and even lake ecology.

Before proceeding with a review of the work on cytoplasmic streaming in the second part of this chapter, we will attempt to highlight those contributions that may be relevant to the role of streaming in cellular metabolism, as well as the experimental techniques that may be of interest in the context of this research.

2.2.1 Cell Models and Surgery Techniques

Characean cells have proven themselves very resilient under manipulation. Over the years, a wide array of techniques has been developed in this system. Some of the more common ones amongst these are:

- *Ligation* – Internodal cells can be truncated by careful ligation with a silk or nylon thread. Ligated cells can survive for several days and streaming continues roughly at *in vivo* rates (Kamiya and Kuroda, 1956; Pickard, 1974).

- *Vacuolar perfusion* – Placing the cell in an isotonic bath (i.e. a solution of an osmolarity similar to that of the vacuolar sap), allows a cell to survive amputation of its ends. If each of the endpoints is then contained in a compartment separated from the rest of the bath, the contents of the vacuole can be replaced by applying a slight pressure difference (Tazawa, 1964; Tazawa and Kishimoto, 1964). This makes it possible to collect the vacuolar sap for analysis and test the effect of changing its make-up on cellular function. After ligation, cells treated in this manner can survive as long as months, depending on the composition of the perfusion-stream. Experiments using this technique are discussed further in section 2.2.3.

- *Tonoplast-free Cell* – The tonoplast of a perfused cell can be destabilised by adding a Ca^{2+} chelator to the perfusion medium, leading to its disintegration. The actin-bundles attached to the chloroplast rows remain intact and the cytoplasm, though disorganised, continues to stream (Shimmen, 2007; Williamson, 1975; Tazawa et al., 1976).

- *Cytoplasm-filled cell* – By gentle centrifugation the cellular cytoplasm can be collected on one end of the cell. Immediate ligation results in a vacuole-free cell that contains only cytoplasm. The protoplasm-filled cell continues to show streaming at rates close to those observed *in vivo* (Kamiya and Kuroda, 1956). In some cases the cytoplasm-filled cells are observed to form new vacuoles.

- *Permeabilised cells* – Bathing the cell in a hypertonic solution induces plasmolysis (i.e. separation of the cytoplasm and membrane from the cell wall due to effusion of water). If the plasma membrane is then treated with a Ca^{2+} chelator, it will be perforated irreversibly and the chemical composition of the cytoplasm can be controlled by exchanging the medium. Cells treated in this manner retain their function to some extent because the cytoplasm is still contained between the intact tonoplast and the perforated membrane (Shimmen, 2007; Shimmen and Tazawa, 1983).

2.2.2 Turgor, Cell Wall Expansion and Growth

Characean internodes have long formed a model system for growth studies, many of which were pioneered in the research of Paul Green and co-workers (Green, 1954; Green and Chapman, 1955; Green, 1958; Green and Stanton, 1967; Green, 1968; Green et al., 1970, 1971; Ray et al., 1972). As discussed in section 2.1.2, the shoot is extended by cell division at the tip, where a dome-shaped *apical cone* divides every three days (see fig 2.2a). The newly formed cell under the apical cone then further divides into a *pro-node* and internode. The latter expands by as much as a factor 30 000 as the cell grows to maturity over a period of about 20 days, attaining a typical length of about 6 cm (Green, 1958).

In the early stages of development, cell expansion is approximately exponential with time (Green, 1954). As the cell matures, this growth levels off, in what is sometimes called a transition to linear growth, finally stabilising to the final cell length. This results in an s-shaped growth curve that is well-represented by a sigmoid function (Dorn and Weisenseel, 1984):

$$H = H_{\infty} \frac{e^{r(t-t_M)}}{1 + e^{r(t-t_M)}} \; . \tag{2.1}$$

Here H_{∞} is the asymptotic length of the fully grown cell, and t_M is the typical time needed for a cell to reach its mid-point in growth. For small times, growth is approximately exponential so that to lowest order:

$$\frac{1}{H} \frac{dH}{dt} \simeq r \; . \tag{2.2}$$

The left side of equation 2.2 is often referred to as the *relative elementary growth rate.*

The interval of cell division, also called the *plastochron*, is quite regular in species like *Nitella Axillaris*. Since internodes form every 3 days, it is possible to estimate the growth rate by comparing neighbouring cell sizes, making the assumption that adjacent cells in a shoot develop in a self-similar fashion. In terms of the cell index *n*, the elementary growth rate can then be approximated as (Green and Chapman, 1955):

$$\frac{1}{H_n} \frac{dH_n}{dt} \simeq \frac{1}{\tau_p} \ln \left(\frac{H_{n+1}}{H_n} \right) \; . \tag{2.3}$$

Studies by Green and co-workers on *Nitella Axillaris* show varying results for the growth pattern. Measurements of a single cell at intervals of about half a day, show an elementary growth rate that falls off from an initial maximum at of about 1 day^{-1} in a fashion more or less consistent with an expansion according to equation (2.1) (Green, 1954). On the other hand, in whole-shoot studies where the growth is estimated using equation (2.3), the elementary rate is found to peak at about 2 day^{-1} roughly one week into development (Green, 1958). It should be noted that the whole-shoot procedure allows observation of the cell size from the point of cell division, when cell lengths are as small as 20 μm, whereas the direct observations start at cell sizes of about 180 μm. This suggests that there is a period of about a week of relatively slow expansion before the regime of exponential growth sets in. A study using *Nitella Flexilis* shows a sigmoid-dependence with $r = 0.3$ (Dorn and Weisenseel, 1984), suggesting that elementary growth rates should be expected to vary significantly between species, and perhaps also according to the experimental protocol.

Positioning a line of markers along the surface reveals that wall expansion is uniform and that a twist develops in the wall as cell grows, causing the markers to follow helical trajectories (Green, 1954). This twist reaches a maximum somewhere during the stage of exponential growth, with the helical wavelength taking on a value as small as $\lambda \simeq 9\,R$. As the cell matures, the direction of rotation slow reverses and the helix unwinds to a typical wavelength around $\lambda \simeq 70\,R$ (Green, 1954; Green and Chapman, 1955).

The direction of cell expansion is pre-dominantly longitudinal and the relation between the radius R and height H is well-approximated by a power-law form $R \propto H^k$, whose exponent is about $k \simeq 0.2$ at 275 K, apparently increasing with temperature (Green and Chapman, 1955).

As is typical in green plants (Taiz, 1984; Cosgrove, 1986), the yielding of the cell wall in characean algae is a turgor driven process. Green and co-workers were the first to investigate the turgor dependence of internodal expansion. Their experiments utilised a micropipette that was fused on one end. A bubble of air is trapped inside the pipette, allowing it to be used as a *micro manometer*. The tip is inserted into the internodal vacuole after which the pressure can be determind by observation of the decrease in bubble size (Green and Stanton, 1967). Using this technique, the growth response to a change in external pressure was then studied through continuous measurements of the s in the presence of external osmotica. The instantaneous response of the cell expansion rate to a change in turgor was roughly in accordance with the following simple form:

$$\frac{dH}{dt} = \begin{cases} m(P - P_c) & P > P_c \\ 0 & P \leq 0 \end{cases} . \tag{2.4}$$

A reduction of the turgor by 0.7 atmospheres was found to completely halt growth, suggesting there is a critical turgor pressure below which growth does not take place. Upon return to normal levels growth transiently increases over a period of about 15 mins, after which it stabilised to previous levels.

Zhu and Boyer (1992) further investigated the influence of turgor pressure on cell growth in Chara Corallina by injecting vacuolar fluid extracted from other cells, thereby changing the concentration of salts, subsequently measuring the growth rate by tethering the end of the cell to a wire connected to a growth transducer. Injection is done by pulses from a large-bore (75 μm) micropipette over a period of 20 min, regulating the pressure with a large-cell probe while the water potential equilibrates to the new osmotic pressure by expulsion of water out of the cell. Inversely, the osmotic pressure can be lowered by extracting vacuolar fluid. Typical turgor pressures are 0.5 MPa. They found that the growth rate abruptly decreases to zero when approaching a threshold turgor P_c (~ 0.4 MPa). At about 110% of the original turgor, a plastic deformation was found to set in, which appeared to damage the cell. Reducing the turgor to normal levels did not result in a return of normal growth. Introduction of external osmotica resulted in more complex behaviour. The combination of transient shrinking and continuation of growth could explain the apparent cessation of growth observed in other studies. Moreover, osmotica were found to inhibit growth, in a way that appeared dependent on the chemical form of the solute. Sucrose and mannitol were more inhibitory than KCl. This was hypothesized to be due to an inhibition of wall enzymes by sugars and analogues.

In further measurements, Proseus et al. (2000) successfully measured the detailed dependence of growth on turgor pressure. Following Ortega (1985), they assumed an equation of the form:

$$\frac{dH}{dt} = m(P - P_c) + \frac{H_0}{\varepsilon_L}\frac{dP}{dt} \,. \tag{2.5}$$

Here $H_0(t)$ is the unstretched cell length and ε_L is the longitudinal component of the elastic modulus. Following Proseus et al. (1999), the elastic prefactor H_0/ε_L is determined by measuring pressure response at a lowered temperature, such that growth is inhibited and expansion is entirely elastic. Subtracting the elastic component from the pressure-response at normal temperature showed a dependence of the steady growth rate on P. This dependence was then measured by series of pressure steps showing a curvilinear response over a range of about 70%-130% of the original turgor pressure. However as reported by Zhu and Boyer (1992), the pressure response above the original turgor pressure was irreversible in the sense that growth rates after reducing P to its original value were always lower than their initial values, indicating some damage had occurred to the cell. Reduction of temperature was also found to decrease the growth rate in a non-reversible manner, with growth rates not returning to their original values after a transient reduction in temperature.

In an extensive set of experiments Proseus and Boyer (2005, 2006a,b,c, 2007, 2008) identified calcium pectate as controlling the rate of deposition of new cell wall material. Experiments in live internodes were compared to isolated cell walls, cut open on one side and glued to a microcapillary,

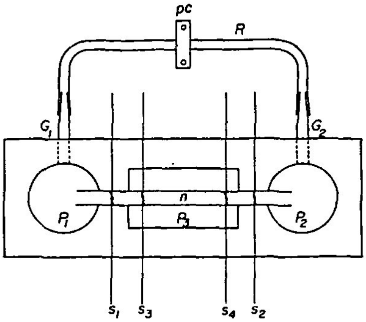

Figure 2.3 The cell holder for perfusion experiments as designed by Tazawa (1964). See text for explanation.

showing a similar curvilinear growth response to pressure in live cells with a strong temperature dependence (Proseus and Boyer, 2006b).

2.2.3 Vacuolar Perfusion Experiments

One of the most studied models of the characean internode is the perfused cell. By placing a cell in a cell holder with 3 isolated compartments containing an isotonic solution, it is possible to gently cut off the ends in the outer compartments, whilst applying a small negative hydrostatic pressure in the central space to prevent collapse. The contents of the vacuole can then be replaced, or *perfused*, after which the cell can be closed off on both ends by careful ligation with a piece of (silk) thread.

Kamiya and Kuroda (1955) were the first to pioneer this process in *Nitella*, reporting survival of ligated cells over several hours. Their procedure was somewhat delicate however, since natural variations between samples make it difficult to exactly match the osmolarity. A hypertonic bathing medium would result in *plasmolysis*, whereas a hypotonic solution could result in cell collapse upon amputation of the ends.

Tazawa (1964) substantially improved on the technique by introducing a cell holder that allowed ligation at two points on each end (see figure 2.3). During amputation, a slight negative turgor was ensured by removing the fluid in the central compartment (P3), letting the remaining film evaporate before cutting off the ends of the cell. This kept the fluid from flowing out at the end and had the additional advantage that it prevented both plasmolysis and collapse when the cell was perfused. To avoid deformation due to crumpling of the cell wall at the ends, the cell was first ligated at the outer points (S1/S2) and allowed to regain its shape in a hypotonic medium before ligating a second time at the inner points (S3/S4). In later experiments (Tazawa and Kishimoto, 1968) the central part was submersed in paraffin to help preserve the cell and improve imaging conditions.

The method introduced by Tazawa resulted in amputated cells that exhibited healthy streaming and could survive for several weeks with a variety of cell sap compositions (Tazawa and Kishimoto, 1964). Cells with a K^+ to Ca^{2+} ratio between 2 and 50 could survive for several days or more, and replacement of K^+ with Na^+, Li^+ or Rb^+ did not adversely affect the streaming rate for at least several days.

Williamson (1975) removed the tonoplast from perfused cells by flushing out most of the cytoplasm with a medium containing EGTA. He found that perfusion caused an abrupt halt in organelle movement after about 60 s, when the tonoplast and most of the cytoplasm covering the organelles had washed away. Organelles then became anchored to actin bundles. Movement could subsequently be reactivated by introducing a 1 mM Na_2ATP solution, with the organelles loosening from the filaments. The rate of movement was close to that observed in vivo. Reactivation was inhibited at high (8.0) and low (6.0) pH. Full streaming rates required a Ca^{2+} concentration of 10^{-7} M or less, 1 mM or more Mg^{2+}, with the Cl^- levels 80 mM or less. Changing the K^+ concentration had little effect. Adding 25 μg ml^{-1} cytochalasin caused a complete halt of the streaming.

Tazawa et al. (1976) reported similar results, finding that a gentle introduction of an EGTA-based medium causes disintegration of the tonoplast, thereby allowing the contents of the vacuole to come in contact with the cytoplasm. More or less in agreement with Williamson, they found that streaming required mM levels of ATP and Mg^2+ were required for streaming, and that Ca^{2+} levels needed to be below 10^{-6} M.

2.2.4 Carbon Fixation and Alkaline-band Formation

Carbon fixation associated with photosynthesis naturally leads to production of OH^-. The reason for this is rooted in the pH dependence of chemical equilibrium of Dissolved Inorganic Carbon (DIC) in water

$$CO_2 + H_2O \leftrightarrows H^+ + HCO_3^- \,. \qquad (2.6)$$

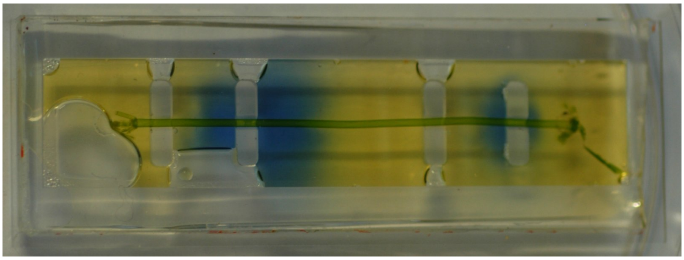

Figure 2.4 Alkaline bands in Chara internode. The cell is placed in a PDMS holder (for design, see section 5.2.1) filled with thymol blue solution. This indicator changes from yellow to blue over the pH range 8.0-9.6. As can be seen, alkaline bands tends to form at the pinching points of the holder, reflecting the fact that membrane mechanics are highly sensitive to mechanical stimulation.

At pH 5.5 roughly 90% of the dissolved carbon is found in the form CO_2, but as the pH increases this balance shifts towards HCO_3^-. At a pH 8.5, which is fairly typical for environments inhabited by characeans, 99% of the inorganic carbon is in the form of HCO_3^-. A proton is therefore required to produce CO_2 from bicarbonate, which inevitably leaves OH^- as a side product. This excess OH^- is generally extruded at the surface, inducing a drop in pH. In characeans OH^- is localised in periodically spaced alkaline bands alternated by acidic regions where H^+ efflux occurs (Tazawa et al., 1987). It has been suggested that the H^+ efflux serves to facilitate photosynthesis by raising the concentration of free CO_2 (Plieth et al., 1994).

Calcium in the form of $CaCO_3$ tends to precipitate on the alkaline regions forming the band-shaped encrustations that lend characeans their colloquial name *stoneworts*. This precipitation yields a proton

$$Ca^{2+} + HCO_3^- \rightarrow CaCO_3 + H^+ \,, \tag{2.7}$$

which may then be used to produce CO_2 from bicarbonate. Evidence that this precipitation directly enhances photosynthetic carbon fixation is found in ^{14}C studies, which show a $1 : 1$ correspondence between the rate of $CaCO_3$ precipitation and the rate of carbon fixation in slightly alkaline environments of pH $8 - 9$ (McConnaughey and Falk, 1991; Mcconnaughey, 1991, 1998).

Further support for the notion the pH bands enhance photosynthesis is found in the work by Mimura et al. (1993), who investigated the effect of the role of the H^+-ATPase on ^{14}C fixation. They observed that carbon fixation was strongly reduced both when the ATP in the cytoplasm was depleted by perfusion with hexokinase and and 2-deoxyglucose, and when the H^+-ATPase was inhibited by introduction of vanadate. Stimulation of the H^+-ATPase by pyruvate kinase and phosphoenolpyruvate (PEP) resulted in increased carbon fixation. The same effects were observed at both pH 5.5 and pH 8.5, indicating that the H^+-ATPase plays an essential role in assimilation of both CO_2 and HCO_3^-.

Evidence that the formation of alkaline bands directely influences the rate of photosynthesis is also found in *PAM fluorometry* studies. When a sample is excited with a fluorescence source, the absorbed light can be dissipated in one of three ways: It can be used for photosynthesis, emitted as fluorescene or dissipated as heat. In practice the degree of heat dissipation can be taken as constant in most samples, so the fluorescence signal can be taken as an inverse measure of the rate of photosynthetic activity. PAM fluorometry measurements show that the external pH correlates directly with the rate of photosynthetic activity, with the highest rates observed in acidic regions (Bulychev and Vredenberg, 2003; Bulychev et al., 2005).

Alkaline band formation appears inseparably linked to cytoplasmic streaming. Inhibition of cytoplasmic streaming with cytochalasin B prevents formation of alkaline bands (Tazawa et al. (1987), citing Lucas and Dainty (1977)). It is therefore not surprising that alkaline bands disappear transiently during an action potential, where streaming is also halted. Babourina et al. (2004) showed that the magnitude of pH variations is greater for the upwards streaming band than the downwards streaming band. Eremin et al. (2007) showed that alkaline bands tend to reform at the same position on the cells surface and the recurrence was increased with the concentration of Ca^{2+} in the surrounding medium.

Given this evidence, the role of streaming in the generation of alkaline bands may be one of the most underexplored aspects its function characean metabolism. The interplay of external pH, membrane transport, streaming, diffusion and photosynthesis found in this context is both rich and complex. Whilst investigations into this subject would constitute a research project on their own and have proven beyond the scope of the project discussed here, they present a truly interesting direction for future work.

2.2.5 Action Potentials

Characean cells exhibit action potentials, a transient depolarisation of the membrane voltage that propagates along both the plasma membrane and the tonoplast. Action potentials in characeans can be triggered by mechanical, electrical or chemical stimulation (Kamiya, 1959; Tazawa et al., 1987) and are a 1000 times slower than those in animals, exhibiting typical propagation speeds of order cm/s (Tabata and Sibaoka, 1987). The bulk rise of membrane conductance underlying this depolarisation results from transient activation of Cl^- and K^+ channels, producing an efflux of KCl into the external medium (Homann and Thiel, 1994; Thiel et al., 1997). The activation of the ion channels is triggered by an increase of free Ca^{2+} in the cytoplasm, with levels rising from ~100 nM to ~1000 nM over a time scale of seconds (Plieth and Hansen, 1996). Streaming is suspended as a consequence of the inhibiting effect of Ca^{2+} on myosin motion and is restored when concentrations return to normal levels (Kamiya, 1959; Tazawa et al., 1987).

Action potentials are hypothesised to play a role in regulating internal pressure. Mechanical stimulation of the cell is known to trigger an action potential through stretch-activated channels in the membrane (Kamiya, 1959; Shimmen, 1996, 1997c,b,a). In salt-tolerant species such as *Chara Longifolia* and *Lamprothamnium*, the same membrane mechanics involved in action potentials are known to play a role in turgor regulation due to changes in external osmolarity, (Stento et al., 2000; Shepherd et al., 2002).

Some controversy exists around the question whether the increased cytosolic calcium levels triggering membrane depolarisation are retrieved from internal stores or obtained from the surrounding medium. Indirect evidence for both scenarios exists. One of the more recent contributions in this discussion is by Plieth et al. (Plieth et al., 1998). Fura-dextran, a fluorescent dye inhibited by Mn^{2+} is injected into the cytoplasm. Because Mn^{2+} is known to pass through the same channels and pores as Ca^{2+} it can be used as a proxy for calcium transport. Injection of Mn^{2+} into the vacuole does not lead to quenching of fluorescence, nor does addition of Mn^{2+} to the external medium. From this the authors conclude that internal stores are the only possible source of Ca^{2+}, indicating the endoplasmic reticulum as their most likely location. Tazawa and Kikuyama on the other hand argue that the evidence indicating release of calcium from internal stores is weak compared to the evidence indicating uptake from the environment (Tazawa and Kikuyama, 2003).

2.2.6 Nutrient Uptake and Intercellular Transport

One of the most obvious ways in which cytoplasmic streaming could benefit an organism is by enhancing the rate of transport between cells, thereby facilitating the translocation of nutrients from regions of uptake to regions of growth. Because of their relative anatomical simplicity, the Charales have been used for a great number of uptake and transport studies. The use of radioisotopes has allowed a extensive measurements of the rate of uptake and transport of salts (Bostrom and Walker, 1975; Williams and Fensom, 1975; Zawadzki and Fensom, 1986a,b) and nutrients such as inorganic carbon (Williams and Fensom, 1975; Zawadzki and Fensom, 1986a,b; Trebacz et al., 1988; Ding and Tazawa, 1989), nitrogen, and phosphorus (Littlefield and Forsberg, 1965; Box, 1986, 1987; Vermeer et al., 2003).

Like most aquatic macrophytes, the Charales are able to take up nutrients from the surrounding water, in contrast to land plants where uptake predominantly takes place in the roots. While the species can successfully be cultured in flasks without needing to be anchored in a layer of soil (Forsberg, 1965), there is evidence that a significant proportion of uptake may take place in the root-like *rhizoidal* cells that anchor the plants, particularly for rate-limiting nutrients such as phosphorus and nitrogen which may be abundant by orders of magnitude in the interstitial water of the sediment (Littlefield and Forsberg, 1965; Box, 1986, 1987; Vermeer et al., 2003).

Dissolved inorganic carbon (DIC) is taken up directly from the surrounding water along the shoot. Low-weight carbohydrates are formed within 1 h after uptake of ^{14}C-DIC and pass unaltered through the plasmodesmata to the neighbouring cell (Trebacz et al., 1988). The bulk of the photoassimilates reside in the endoplasmic layer on the inside of the chloroplasts, but particularly in branchlet cells some fraction is sequestered in the vacuole (Ding et al., 1991b). The branchlet cells are more highly photosynthetically active than the internodes, and transport from the branchlets to internodes can be five times higher than transport in the reverse direction (Ding et al., 1991b,a).

Intercellular transport is know to show a seasonal dependence (Zawadzki and Fensom, 1986a), investigated in detail by Shepherd and Goodwin (1992a,b). In winter time, the plants are in a dormant state characterised by a low plasmalemma potential difference (−120 mV) and low rates of intercellular communication. In spring and summer the plasmalemma potential increases to almost twofold and intercellular transport, as observed by injection of fluorescent probes, increases significantly. It is observed that action potentials reduce the flux between cells. Inhibition of Ca^{2+} channels by application of La^{3+} increases transport, which is also naturally higher in cells not susceptible to action potentials.

A number of studies have investigated the relationship between streaming and intercellular transport. Studies with a tandem pair of internodes show that the transport rate correlates with the streaming rate over the 25% variation in magnitude observed over a collection of samples (Zawadzki and Fensom, 1986a). This correlation is stronger in summer time when the rate of transport is higher. Treatment with Cytochalasin B shows a roughly proportional reduction of the transport rate with the streaming velocity (Bostrom and Walker, 1976). Treatment of either cell in the tandem pair suffices to lower the transport rate and a similar response is observed when altering the streaming velocity by lowering the temperature Ding and Tazawa (1989). Some early studies found indications of a small component of propagation faster than the streaming velocity (Williams and Fensom, 1975; Dale et al., 1983). This finding was not confirmed by later whole shoot experiments where ^{14}C is fed to the rhizoids showing that carbon is transported upwards at roughly the rate of streaming, while 32P transport is slower but of a similar magnitude (Box et al., 1984).

2.3 Rotational Streaming in the Charales

2.3.1 Rate of Streaming and Velocity Profile

Rotational streaming in characeans is one of the fastest motor-driven processes known in cells. Depending on cell type and species, velocities reported range from 10 μm/s to 100 μm/s, with 60 μm/s often being quoted as a typical velocity. Over the last 5 decades experiments have probed many aspects of streaming. Techniques used for measuring typically fall into the categories:

- *Direct observation* – Through light microscopy, either bright field or Differential Image Contrast (DIC). Usually particle speeds are determined by timing intervals with a stopwatch (Kamiya and Kuroda, 1956).

- *Micro-cinematography* – Light microscopy is recorded to film and particles are subsequently tracked visually using a motion analyser (Allen, 1974).

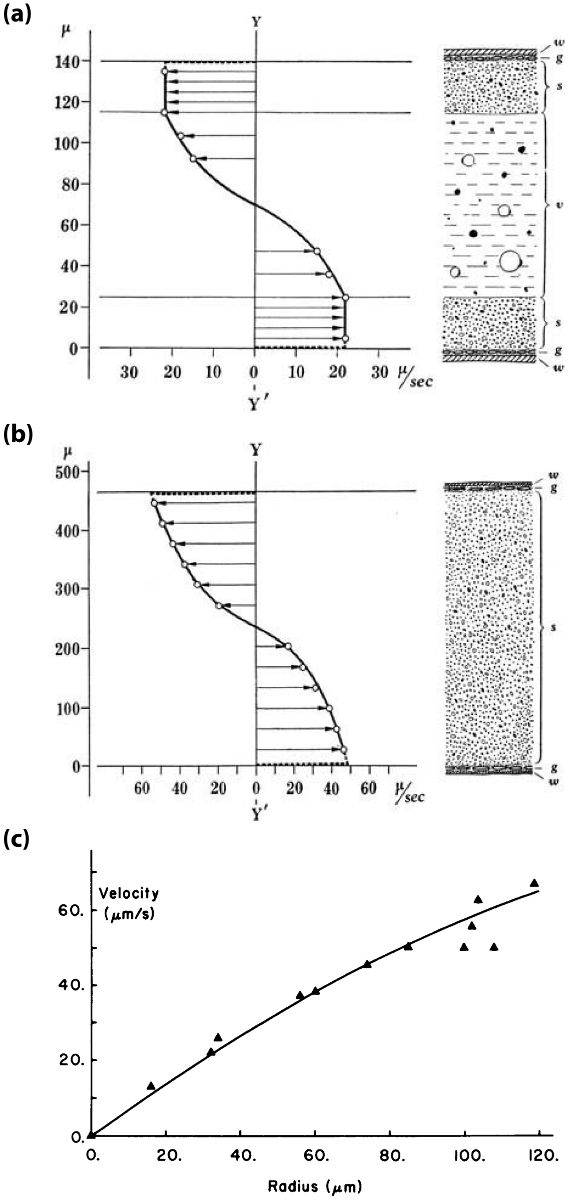

Figure 2.5 Flow profiles as measured by Kamiya and Kuroda (1956) and Mustacich and Ware (1977). **(a)** Measurements inside a 'leaf' cell from one of the branchlets Kamiya and Kuroda (1956). **(b)** Measurements of a protoplasm filled cell created by centrifugation and ligation (Kamiya and Kuroda, 1956). **(c)** Laser Doppler velocimetry measurements inside an internodal cell as presented by Mustacich and Ware (1977).

- *Laser Doppler Velocimetry* – A laser beam is focused on the cytoplasm. From the Doppler shift in the scattered light, a mean velocity and spread can be determined (Mustacich and Ware, 1974).

The best known direct measurements of the wall-to-wall profile are those reported by Kamiya and Kuroda (1956), who measured profiles in three types of cells: (1) rhizoidal cells, found at the base of the plant, (2) 'leaf' cells sprouting from nodes and (3) and protoplasm-filled internodal cells created by the method described above. Measurements show a constant velocity within the cytoplasm and a curved shear profile within the vacuole. In leaf cells, which have a straight (i.e. non-spiralling) neutral lines, the shear profile is compatible to measurement precision with axially directed driving at the walls. The profiles obtained are shown in figures 2.5a-b.

Mustacich and Ware (1977) improve on the Kamiya and Kuroda measurements by using laser Doppler scattering. In order to improve visibility, the region of interest is exposed to a high-intensity beam from an argon laser prior to observation. This causes chloroplasts to swell and ultimately detach. Streaming is restored to former levels after one or two days (for this technique, see also Kamitsubo (1972); Allen (1974)). A centre-to-wall profile is presented that has superior resolution to the Kamiya data (fig 2.5c).

Pickard (1972) presents measurements across a full cross-section of an internodal cell in *Chara Braunii*. He also obtains a closed form solution to the fluid equations of motion by approximating the internodal flow as axially symmetric, thereby neglecting higher order effects due to the spiralling. In this case, solving Stokes-flow effectively reduces to solving Laplace's equation on a circle (for details, see chapter 3), and the solution of the form $v_z \sim \sum W^n r^n sin(n\theta)$ is obtained readily by separation of variables. Assuming step boundary conditions allows a closed-form solution:

$$v_z = \zeta(r,\theta) = \frac{2V}{\pi} \arctan \frac{2(r/R)\sin\theta}{1-(r/R)^2} \tag{2.8}$$

We will discuss this solution further in chapter 3, where we discuss our own helical generalisation of this flow problem. Pickard plots $\zeta(r,\theta)$ against 115 measurements of v_z, showing good correspondence, further establishing the idea put forward by Kamiya and Kuroda that flow in the vacuole can be approximated as a simple Newtonian shear flow with imposed velocities at the boundaries.

2.3.2 Dependence on Cell Size

Few authors discuss the scaling of the streaming velocity with cell size. At the time of writing, the only work available to the author is that of Pickard (1974), who in turn cites Hayashi (1952). Pickard investigates the dependence on cell length by shortening long cells by ligation, measuring the original and final streaming rate each time. His results indicate that streaming velocities become independent of cell length for aspect ratios larger than 5. He also shows a quite general scaling $V^{-1} \sim D^{-1/2}$ between the streaming velocity V and the cell diameter D, that holds for various cell types in Chara Braunii. He proceeds to show that his results are compatible with the simple assumption that the power per unit area applied to the cytoplasm is a constant independent of streaming velocity and radius.

2.3.3 Dependence on Temperature

Multiple studies have measured the dependence streaming on temperature (Kamiya, 1959; Tazawa, 1968; Pickard, 1974; Mustacich and Ware, 1976). Typically the rate of streaming increases with temperature, with characeans commonly showing a linear dependence. There exists a *supra-optimal* temperature above which streaming ceases and cell death follows. Some authors also report a minimum temperature below which streaming halts. Pickard (1974) reports an range for *C. Braunii* of $5 - 45$ C. Mustacich and Ware (1976) report $4 - 34$ C for *N. Flexilis*. The data presented in Kamiya's 1959 review shows non-zero streaming at temperatures as low as 2 C for *N. Syncarpa* (Nägeli, 1860) and as low as 1 C for *N. Mucronata* (Lambers, 1925), though we have not located the original sources to check the protocol used. The dependence of streaming on temperature is significant. Mustacich reports $2.6\ \mu\mathrm{m\ s^{-1}\ K^{-1}}$ (Mustacich and Ware, 1976) and Pickard (1974) reports $3.4\ \mu\mathrm{m\ s^{-1}\ K^{-1}}$. This means that measurements of streaming velocities should always take temperature into account, as differences of a few Kelvin correspond to significant changes in streaming velocity. In some cases it has also been observed that a sudden jump in temperature, either up or down, halts streaming temporarily, though not all results are in agreement (Kamiya, 1959).

2.3.4 Light Sensitivity

Visible light can retard, stop or accelerate streaming, and in some cases induce streaming in inactive cells (Kamiya, 1959). Plieth and Hansen found that the transition from dark to light causes a reversible increase in the streaming rate of 15 – 30% (Plieth and Hansen, 1992), with a typical time constant of 300 s for adjustment. The transition from light to dark was much slower and could take as much as 2000 s. Harvey observed in *Nitella* that a high intensity UV flash briefly stops streaming locally, and only on the side of the cell directly exposed (Harvey, 1942). Mustacich and Ware showed that a highly focused beam from an Argon laser (514.5 nm) inhibits streaming locally, affecting a region from 200 μm upstream to 400 μm downstream. As mentioned earlier, prolonged exposure from any high intensity light source is known to damage the chloroplasts, which swell and ultimately detach. Streaming is restored after a few days of recovery and this technique is used commonly to create a chloroplast-free window for observation of streaming, especially when using laser-Doppler scattering (Kamitsubo, 1972; Allen, 1974; Mustacich and Ware, 1974, 1976, 1977; Ackers et al., 1994).

2.3.5 Gravity Sensing

Vertically oriented cells typically show a 1.10 ratio in the upward (*basipetal*) and downward (*acropetal*) streaming rates that is well documented for many characean algae (see Staves et al. (1997) and references therein). Because this difference in velocity is not present in a horizontally oriented cell, cytoplasmic streaming has been implicated in connection to gravity sensing in characean cells, which in turn plays a role in growth control.

Staves, Wayne and Leopold claim that the difference in streaming velocities is a result of physiological control, rather than a direct effect of gravity (Wayne et al., 1990, 1992; Staves et al., 1992, 1995, 1997). Differential pressure exerted by the cytoplasmic mass on the extra-cellular matrix (ECM) is hypothesised to act as the sensing mechanism. A rivalling theory attributes gravity sensing to sedimentation of *statoliths* and parabolic flight experiments have even be performed to test this idea (Limbach et al., 2005; Volkmann et al., 1991). However, the fact that the streaming difference disappears when the density of the surrounding medium is matched to that of the bulk protoplasm appears to discredit statolith theory (Staves et al., 1997).

2.3.6 The Actin-myosin System

Identification of the Streaming Mechanism in Characeans

Since *sliding theory* was proposed by Kamiya and Kuroda (1956) to explain the lack of shear gradient observed within streaming cytoplasm, five decades of research have lead to the understanding that cellular organelles are ferried along bundles of actin filaments fixed on the chloroplast rows by myosin XI molecular motors. Good overviews of the research leading to the isolation of the actin-myosin system in characeans are given by Shimmen (Shimmen and Yokota, 2004; Shimmen, 2007).

The identification of actin started with the work of Kamitsubo (1966), who found cables on the surface of chloroplasts and developed the technique for creating a chloroplast-free window (Kamitsubo, 1972). The fact that streaming along the chloroplast-free window was restored once the cables regenerated formed a direct indication of their involvement in cytoplasmic streaming. Electron microscopy observation by Nagai and Rebhun (1966) showed that the cables were in fact *bundles* of microfilaments. Evidence that the microfilaments were a form of actin was presented by Williamson (1974) and Palevitz et al. (1974), who demonstrated that *heavy meromyosin* (HMM) attaches to microfilaments, first in extracted cytoplasm and later also *in situ* on the chloroplast rows (Palevitz and Hepler, 1975). Kersey et al. (1976) subsequently showed that the actin bundles are polarised and that the direction of streaming corresponds to the polarisation of the filaments.

Using tonoplast-free cells Williamson (1975) and Tazawa et al. (1976) demonstrated that streaming requires ATP and is inhibited by cytochalasin B, which prevents actin polymerisation. The work by Williamson also suggested that a myosin-like component attaches to cellular organelles, as organelles became strongly bound to the actin bundles in an ATP-depleted perfusion stream. Nagai and Hayama (1979) used electron microscopy to show that this linkage was achieved through nm-scale regularly spaced electron-dense structures. Kachar and Reese (1988) later found that a 3-dimensional membrane structure believed to be the rough endoplasmic reticulum moves along the actin filaments. Shimmen and Tazawa (1982) successfully reintroduced centrifuged cytoplasmic organelles from *Chara Australis* in a tonoplast-free cell of *Nitella Axilliformis*, which then moved along the direction of polarisation , providing further suggestion of that cellular organelles associate with myosin. Purification of myosin proved technically difficult, but was ultimately achieved for lily pollen tubes by Yokota and Shimmen (1994) and for *Chara Corallina* by Yamamoto et al. (1994).

Motile properties of Characean Myosin

Motion of myosin along actin filaments has been studied using a variety of *in vitro* and hybrid motility assays throughout the years. The first generation of these was developed by Kuroda (1983), who cut open a *Nitella* internodal cell and spread the cell wall with intact chloroplast rows out on a cover slip, to create a plane of actin tracks. A slow reconstituted streaming of about 0.4 μm/s was observed in the presence of HMM. Sheetz and Spudich (1983) used the same technique to measure the movement of HMM-coated fluorescent beads, thereby creating the first *in vivo/in vitro* hybrid motility assay. The average bead velocity observed was 2.5 μm/s. Yamamoto et al. (1982) developed a method to treat actin from *Dyctiostelium* with biotinylated *severin*, so that it could be attached to a substrate coated with *avidin*. Using this method, HMM-coated beads could be observed moving along the filaments at rates similar to the *Nitella* system. Kron and Spudich (1986) later visualised motion of *rhodamin-phalloidin* labelled actin along cover slips coated with rabbit skeletal myosin, observing rates of motion of 3 – 4 μm/s. Kohno et al. (1991) applied this technique to a crude extract from lilly pollen tubes and observed sliding at 2 μm/s. A motility assay with myosin extracted from *Chara Corallina* was finally constructed by Yamamoto et al. (1994), who observed rates of motion of 25 μm/s. Higashi-fujime et al. (1995) subsequently observed average velocities around 60 μm/s using a similar assay, a velocity that is of the same magnitude as the streaming velocities observed *in vivo*.

The collective force-velocity relation of streaming motion was probed using a centrifuge-microscope by Oiwa et al. (1990), Chaen et al. (1995) and Sugi and Chaen (2003), who measured the velocities of myosin-coated polystyrene beads injected into the cytoplasm. Beads with large (> 8 pN) stall forces displayed a linear decrease of the velocity as the force was increased towards the stall force. Beads with small stall forces (< 2.4 pN) displayed a double-hyperbolic curve, analogous to the force-velocity curve of single muscle fibres.

Stepping dynamics of characean myosin have been investigated with motility assays and single molecule optical trapping. Some controversy still exists around the questions how the high speeds of motion are achieved and whether stepping is *processive* (i.e. occurring in continuous stretches) or *non-processive* (i.e. with detachment following each step).

Awata et al. (2003) presented motility assay studies that showed that a minimum density of 200 myosin molecules per μm^2 is required to induce sliding. Comparison with similar studies using processive motors, which require a density of only 1 myosin per μm^2, suggests that characean myosin moves in a non-processive manner (i.e. motion occurs in discrete steps, rather than continuous stretches). This non-processivity was also supported by single-molecule studies by Kimura et al. (2003), who found a non-processive motion with a step size of 19 nm. Sumiyoshi et al. (2007) contradict these findings and claim that motion might well be processive and that the step-size should be expected to be close to the half pitch of an actin filament strand, which is 35 nm. They base this hypothesis on the fact that they measure a high duty ratio of the cycle, which translates to long attachment times. This high duty ratio is further supported by the observation of pivot motion of the myosin molecules. They claim this motion could not occur under physiological conditions at the duty ratio presented by Awata et al. (2003), which is smaller by a factor of ten. The non-processivity of myosin in characeans is further called into question by work on myosin XI in higher plants, which shows that motion is processive, with a step-size of 35 nm.

A number of explanations for the high speeds of characean myosin have been put forward. Yamamoto and colleagues hypothesise that the high ATPase activity of up to 500 s^{-1} $head^{-1}$ (Ito et al., 2003) and low duty ratio (Awata et al., 2003) conspire to produce a high stepping frequency. It should be noted that these high rates of ATP consumption would quickly deplete supplies if all myosin molecules present in the cytoplasm are fully activated. Yamamoto et al. (2006) estimate that an activated fraction of order 0.01 is sufficient to sustain streaming. Sumiyoshi et al. (2007) again contradict the Yamamoto group hypothesis, claiming that the tenfold larger duty ratio found in their work is incompatible with the high stepping rates proposed. Kimura et al. (2003) in turn note that the measured dwell time (i.e. the dead time after a step) under typical loads is too long to allow the high frequencies required to attain *in vivo* velocities. They therefore propose a cooperative effect, where the step of a single motor produces a negative load on other motors that could increase the rate of ADP release in the post-force generation state and shortening the dwell time.

2.3.7 Driving Mechanics and Cytoplasmic Rheology

Various studies have investigated the driving mechanism and rheological aspects of cytoplasmic streaming. A number of authors have presented theoretical models of transport along the cytoskeleton with stochastic on-off dynamics (Dinh et al., 2006; Snider et al., 2004; Maly, 2002; Smith and Simmons, 2001). The most recent work of this form applied to streaming is by Houtman et al. (2007), who describe streaming in transvacuolar strands with a 2D model that includes on/off kinetics and hydrodynamic interactions between particles through an Oseen tensor.

There is also a range of studies that focus on the driving force associated with streaming. As mentioned in 2.3.2, a study by Pickard (1974) investigates the scaling of streaming with cell size. He presents analysis to support the notion that most dissipation should occur near the neutral lines. As metioned in section 2.3.2, his measurements of the streaming velocity as a function of cell size show a $V^{-1} \sim R^{-1/2}$ scaling. He notes that this is consistent with the combined assumption of a dissipation rate scaling as V^2 and a driving power scaling as R.

Tazawa and Kishimoto (1968) used perfusion experiments (see section 2.2.3) to measure the motive force. The pressure difference between the two reservoirs is adjusted so that streaming is halted in one of the bands, implying that the shear force balances the motive force in the cytoplasmic layer. The motive force obtained this way has a value in the range of 14 – 20 μN/cm^2, consistent with centrifuge microscope measurements (Kamiya and Kuroda, 1958), and is independent of temperature, suggesting that the temperature-dependence of streaming is a consequence of a change in cytoplasmic viscosity. He also examines the effect of tonicity, finding an increase in the tonicity from 290 mM to 586 mM results in a lowered streaming rate as well as an increased motive force. Decreasing the tonicity in the cytoplasm to 190 mM resulted in a swelling of the chloroplasts, causing a marked decrease in streaming rate and motive force, presumably because of a resulting deformation in the actin bundles. After 5 – 20 mins, the chloroplasts regained their normal shape and streaming recovered, though the motive force remained lowered.

Donaldson (1972) performed measurements of the forward streaming velocity as a function of the applied perfusion pressure gradient. He assumes a driving force localised to a layer thickness ϵ and a power-law dependence $\tau = -\alpha(\partial u/\partial y)^{1/n}$ for the viscous stress. His results show a good correspondence for $n = 3$ and $\epsilon = 0.1$ μm. The corresponding motive force is $F = 36\ \mu\text{N cm}^{-2}$ is higher than the values found by Kamiya and Kuroda (1958) and Tazawa and Kishimoto (1968), which he concludes is the result of a a systematic underestimation in these measurements due to the fact that the small velocities and thin layers of movement near the stalling point are very difficult to observe.

Hayashi (1980) applies similar analysis to the experimental results from Kamiya and Kuroda (1956) for a protoplasm-filled cell. He approximates the protoplasm, which is non-Newtonian and viscoelastic, as a power-law fluid and determines the best fit parameters for the protoplasm filled cell, as well as two cases of plug flow of extracted cytoplasm (Kamiya and Kuroda, 1963). He finds an exponent of $n = 1.4$ for the protoplasm-filled cell and exponents of $n = 1.3$ and $n = 1.7$ for the two cases of plug flow.

Nothnagel and Webb (1982) investigate various hydrodynamic models for driving mechanics in the cytoplasm. They conclude that the virtually shear-less profile observed in the cytoplasm is best explained by assuming a meshwork throughout the cytoplasm that is pulled along at the cell wall. The endoplasmic reticulum is argued to be a structure that could fulfil such a role and there is some structural evidence from electron micrographs to support this hypothesis (Kachar and Reese, 1988).

The role of the ER as backbone appears not to have been fully accepted in the community. Indeed, given the large number of fluid-like materials the exhibit yielding behaviour (Coussot et al., 2002), any number of alternative rheological models may well describe the gel-like movement of the cytoplasm.

2.4 Possible Implications for Biological Function

Although a great corpus of work has been published on the molecular basis and even hydrodynamics of streaming, relatively few authors venture into a discussion of its function. It has long been suggested that streaming aids molecular transport in some way. Kamiya's 1959 review mentions that de Vries argued to this extent as early as 1885. However, concrete hypotheses as to the mechanism by which streaming accelerates metabolic rates have scarcely been put forward. Agutter et al. have argued that diffusion is not capable of explaining many transport phenomena in cells (Agutter et al., 2000; Agutter and Wheatley, 2000). Similarly, Hochachka (1999) presents an argument that the degree of homoeostasis along ATP pathways cannot be explained other than by assuming forms of active transport.

The most important contributions to this discussion are without doubt from Pickard. He discusses scaling of the streaming velocity and diffusional time scales with cell size (Pickard, 1974). He points out that a point-source in the streaming cytoplasm will become a line-source w.r.t. objects fixed at the cell wall. He also suggests that streaming as such does not necessarily have to confer a benefit to the cell if its real purpose is transport of particles along the cytoskeleton. This second point is argued further in a later publication where Pickard makes a case for streaming being an accidental consequence for vesicular transport along the cytoskeleton (Pickard, 2003). Finally he discusses scaling of exchange of molecular species between organelles and their environment in advection-dominated flows, where the Péclet number, which measures the dimensionless ratio of advective and diffusive transport, is high (Pickard, 2006). He shows that uptake into a sphere scales as Pe^2 at moderate Péclet numbers and crosses over to $\mathrm{Pe}^{1/3}$ for large values of Pe. The underlying ideas are very similar to those discussed in section 1.3 in the context of the swimming of *Volvox*, a spherical colony of algae containing as much as 2000 cells, that generates high Péclet number flows in order to increase its maximum rate of nutrient uptake (Solari et al., 2006; Short et al., 2006).

Taking a broader look at the material presented in this review, we can identify a range of metabolic processes where streaming may play a role.

- *Intercellular transport* – One of the most obvious ways in which a streaming system might benefit the organism is the enhancement transport between cells. As we have seen in section 2.2.2, the characean algae show rapid growth by repeated division of a cell at the tip. Nutrient uptake and intercellular transport studies (sec 2.2.6) show that characean algae take up nutrients from the water along their shoots, as well as from the soil through root-like *rhizoidal* cells. It is also known that the most active photosynthesis takes place in the branchlets. There is evidence that some forms of transport are polar in nature, i.e. have a directional preference from roots and branchlets to the tip (Ding et al., 1991a,b), and the streaming rate has been shown to affect intercellular transport in a number of studies (Bostrom and Walker, 1975, 1976; Zawadzki and Fensom, 1986a; Ding and Tazawa, 1989).

Streaming therefore clearly aids intercellular transport, and perhaps this is in fact the main reason for its existence. The movement of the cytoplasmic bands could serve simply to act as a form of conveyor belt to speed up transport of nutrients and metabolites towards the tip where they are needed for growth. The exact benefits streaming affords in this manner are not so straightforward to quantify. What is known about the rate of transport through the plasmodesmata suggests that fluxes are effectively diffusive. The influence of streaming on the rate of transport would therefore likely involve the details of boundary layer dynamics near the plasmodesmatal openings, where too little is known to develop theory. For certain basic metabolites, such as inorganic carbon, it may be possible to estimate the nutrient requirements of the growing apex to assess how much streaming must facilitate transport between cells in order to allow the growth observed *in vivo*.

- *Enhancement of cytoplasmic diffusion* – Pickard (1974), among others, raises the notion that streaming could enhance mixing within the cytoplasmic layer. This enhancement of diffusive transport would have to arise either from gradients in the mean velocity field or from temporal fluctuations in the local velocity. What is known about the flow field and cytoplasmic rheology suggests these gradients are small, with the exception of the regions near the neutral line. The Rheological evidence (sec 2.3.7) shows that the sheared boundary layer near the wall could be as thin as 0.1 μm (Donaldson, 1972), suggesting that inhomogeneities near the wall are very localised. Moreover, given that the thickness of the cytoplasm is of order 2% of the cellular radius, the amount of vacuolar shear across the cytoplasmic layer should be expected to be < 1 μm/s. For medium sized proteins this yields an upper bound estimate $\mathrm{Pe} = \Delta U a/D \leq 1$ for the Péclet number of the cytoplasmic flow. This implies that the level of shear is likely insufficient to induce Taylor dispersion in the cytoplasmic layer, since the original work on pipe flows (Taylor (1954b), see also section 4.2) indicates that this type of dispersion takes place in the limit $4L/a \gg \mathrm{Pe} \gg 6.9$. Moreover, it is possible that shear is further constrained by the endoplasmic reticulum, whose mesh-like structure extending throughout the cytoplasmic layer could have a gelling effect on the rheology (Kachar and Reese, 1988).

It is therefore not immediately apparent how the mere fact that the cytoplasm is moving would affect diffusion of metabolites. It could be that there are temporal fluctuations in the cytoplasm that aid mixing. However, little data exists on the magnitude of these fluctuations. The laser Doppler scattering experiments yield some information about the variability of velocities, but it is difficult give a precise interpretation to these results. Direct measurements of these fluctuations have proven challenging. The variations in particle velocities are hard to separate from the experimental error. A further complication is that the thickness of the cytoplasmic layer varies significantly over time. We can therefore not take the variations in velocity at any fixed point in the sample as a measure of the velocity fluctuations experienced by an advected parcel over time.

- *Flow past chloroplasts* – A significant fraction of metabolic activity is localised to the stagnant *periplasmic* layer that holds the chloroplasts and mitochondria. With respect to the metabolism of these organelles, the simple fact that cytoplasm moves along the chloroplast rows should aid in maintaining homoeostasis to some extent. As a result of this, the immobile layer near the wall effectively interacts with a larger volume of cytoplasm, to a degree that should in principle increase with the Péclet number. This idea is akin to the notion presented by Pickard (1974) that a point source effectively becomes a line source in a flowing environment. The extent of this effect is limited in the sense that only fluctuations in the direction of flow will be suppressed. Circumferential enhancement of diffusion will be minimal, except possibly at the neutral lines.

- *Alkaline band formation and photosynthesis* – As indicated in section 2.2.4, there appears to be a connection between streaming, alkaline band formation and photosynthesis. A number of studies point to the fact photosynthetic carbon fixation is enhanced by alkaline band formation and the Calcium precipitation that results from it, whilst other studies show that streaming is required for the formation of these bands. This suggests that streaming directly ties into the ability of the organism to extract carbon from its environment. The complexity of this problem, that involves an interplay of external pH, membrane transport, streaming, diffusion and photosynthesis, easily warrants an entire study to itself. As such we have found it to be beyond the scope of this first study, but it remains a key direction for future research.

The Role of Vacuolar Mixing in Cellular Metabolism

While it is clear that the of ideas presented above warrant further investigation, the question that any of these explanations fail to offer insight into, is why the geometry of flow exhibits such a strikingly symmetric form. In particular, why is streaming up and down the cell the separated into only two bands, and why are these bands helical in shape? It is this aspect of geometry that we will attempt to gain insight into by exploring the hydrodynamics of the streaming flow.

One of the perhaps most significant implications of the two-band design found in Chara is that it produces a shear flow that extends all the way into the vacuole — a higher number of more narrow bands would certainly produce flows more localised to the periphery. Few insights have been offered as to the metabolical significance of this vacuolar shear. The vacuole has in the past often been regarded as a relatively insignificant to cellular metabolism. However, over the last decades it has become increasingly clear that the vacuole fulfils a multitude of metabolic roles, acting as a buffering reservoir for pH homeostasis, and storing various metabolical building blocks such as sugars, polysaccharides and and organic acids (Taiz, 1992). As such transport between vacuole and cytoplasm, and the mixing of the vacuolar contents may well be of greater significance than previously realised. In Chara and Nitella species, the cellular radius is one to two orders of magnitude larger than in typical plant cells. Vacuolar transport may therefore well be diffusion-limited for certain processes, since diffusional time scales increase quadratically with the length scale and may thus be up to 4 orders of magnitude slower than in smaller cells.

In this context the helicity of the system may prove significant. As discussed in the section 2.2.2, the twist of internodal cells is known to evolve over the duration of cell development (Green, 1954; Green and Chapman, 1955). During early stages of growth an increasing twist develops in the cell wall, followed by a gentle untwisting as the cell expands towards its mature length. This kind of controlled evolution suggests that there may be a relevance to the helicity of the bands, particularly in the middle phases of growth when a steady supply of nutrients is critical. This raises the question whether the helicity of this system could serve to internal mixing in some shape or form.

In the next chapter we present a simplest hydrodynamic description of the internodal flow and find a natural decomposition into two components. In addition to the dominant component parallel to the helical forcing, we find a small secondary component transverse to the helical axis. That component takes the form of a circulation between the two neutral lines. It is this second component that has fundamental implications for bulk transport of molecular species. As we will argue in chapter 4, this could imply that one function of streaming is to enhance diffusional transport into and out of the vacuole.

3 HYDRODYNAMICS OF CYTOPLASMIC STREAMING

Given the complexity and energetic cost of of having a streaming system, it seems clear that cyclosis must confer some significant benefit to the organism. In the last chapter we have seen that streaming is indeed implicated in variety of processes. The most obvious of these is perhaps intercellular transport. Since Charales exhibit growth at the tip, yet are able to take up nutrients and perform photosynthesis all along their surface, there is a clear utility to the transportation of nutrients and cellular building blocks up along the organism. Additionally, streaming may facilitate the formation of alkaline bands, which in turn appears to enhance carbon uptake. These aspects certainly merit further investigation, but in order to narrow down the scope of the current project, we will begin with a closer examination of the hydrodynamics of the internodal flow and their relation to intracellular mixing.

The main aspect that we will focus on in this work, is the question how streaming could help overcome the disadvantages associated with having cells of such a large size. As discussed previously, the notion has often been expressed in the literature that streaming somehow enhances mixing, thereby aiding *homeostasis*, the stabilisation of metabolic rates over fluctuating levels of uptake and demand. Yet few mechanisms have been put forward whose benefits can be quantified. It is our goal to examine the fluid mechanics of this circulation with the hope of elucidating in what way it may contribute to enhancing metabolic rates.

In searching for quantitative descriptions of the benefits of streaming, the characean algae present something of a unique case. The flow in these organisms is perhaps the most organised and symmetrical of all the streaming types found in nature. Two distinguishing features of the Charales that require particular explanation are:

1. The reason for the high streaming rates found *in vivo*, which could presumably only have evolved as a result of considerable genetic pressure.

2. The reason for having a helically directed shear flow that extends into the vacuole.

As discussed in the previous chapter, it is not immediately obvious in what way streaming could enhance mixing within the layer of cytoplasm at